Available online at www.sciencedirect.com
SCIENCE DIRECT®

Veterinary Immunology and Immunopathology 103 (2005) 53–65

Veterinary immunology and immunopathology

www.elsevier.com/locate/vetimm

Proteomic survey of bovine neutrophils

John D. Lippolis*, Timothy A. Reinhardt

Periparturient Diseases of Cattle Research Unit, USDA-ARS, National Animal Disease Center, Ames, IA 50010, USA

Received 14 June 2004; received in revised form 3 August 2004; accepted 23 August 2004

Abstract

Mastitis is a major economic concern for the dairy industry. Conditions such as parturition cause a transient immunosuppression that leads to increased incidence of mastitis. One facet of periparturient immunosuppression is a functional impairment of the blood and milk neutrophils in dairy cows. To better understand the biology of the bovine neutrophil we report the first proteomic analysis of the bovine neutrophil. We have identified over 250 proteins using one-dimensional electrophoresis followed by reverse-phase chromatography in line with electrospray tandem mass spectrometry. A large number of metabolic proteins were identified, including most of the enzymes required for generation of NADPH and ATP. In addition, many proteins were identified that participate in cell mobility and phagocytosis. All the bovine members of the cathelicidin family were identified, as well as other proteins with immunological functions. Proteins important for cell signaling, vesicular transport, control of apoptosis and other functions were identified giving an overview of the bovine neutrophil proteome.
Published by Elsevier B.V.

Keywords: Neutrophil; Proteomics; Bovine

1. Introduction

Polymorphonuclear neutrophil leukocytes provide a first line of defense against invading pathogens at the site of infection (Mollinedo et al., 1999; Paape et al., 2003; Smith, 2000; Zychlinsky et al., 2003). The importance of neutrophils in pathogen control within the dairy cow mammary gland is highlighted by the observation that diminished neutrophil function is concomitant with increased incidence of mastitis (Paape et al., 2002). Periparturient dairy cows have been shown to have significant neutrophil functional suppression that is associated with increased prevalence of mastitis (Kehrli et al., 1989; Mehrzad et al., 2001; Shuster et al., 1996). Understanding the causes of this immunosuppression is of great importance to the dairy industry.

Advances in mass spectrometry have led to an increased understanding of the protein expression profile for various cell types. Due to the observation that there is often a poor concordance between mRNA transcript and protein levels, we have focused our efforts on elucidating the neutrophil proteome. Mass spectrometry is ideally suited for the determination of

* Corresponding author. Tel.: +1 515 663 7446; fax: +1 515 663 7669.
E-mail address: jlippoli@nadc.ars.usda.gov (J.D. Lippolis).

0165-2427/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetimm.2004.08.019

the protein profile of various cell types (Aebersold and Mann, 2003; Yates, 2004). Proteomic analysis of neutrophils has been reported in human and rat. Boussac and Garin limited the scope of their work to proteins secreted by neutrophils in a calcium-dependent manner, and therefore, the number of proteins was limited (Boussac and Garin, 2000). Piubelli et al. identified 52 major protein spots from two-dimensional gel electrophoresis of rat neutrophil. However, several of these spots represent multiple post-translational modifications of the same protein (Piubelli et al., 2002). Fessler and coworkers presented data only on proteins that changed in expression level between a stimulated and non-stimulated state (Fessler et al., 2002). This report presents in greater detail an analysis of the proteome of circulating neutrophils and specifically the bovine neutrophil.

One of the challenges in generating a protein profile of a cell type is the complexity of the proteome. Several strategies have been developed to increase sensitivity by fractionation of the sample by various methods (i.e. strong cation exchange, one-dimensional and two-dimensional gel electrophoresis) prior to reverse-phase chromatography directly in line with the electrospray tandem mass spectrometry (Aebersold and Mann, 2003). In this work we use one-dimensional gel electrophoresis, followed by in-gel trypsin digestion, prior to mass spectrometry (Fig. 1). This approach yielded the identification of greater than 250 proteins that represent the abundant proteome of a circulating bovine neutrophil.

2. Materials and methods

2.1. Isolation of neutrophils and protein preparation

Blood was collected from six healthy dairy cows an average of 27.5 days prior to their second calving (3–4 years old) by jugular venipuncture. Neutrophils isolated from packed red blood cells by hypotonic shock (Roth and Kaeberle, 1981). 1×10^7 neutrophils from each cow were combined and homogenized in 10 volumes of a buffer containing: 10 mM Tris–HCl, 2 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.25 M sucrose, 4 ng/ml aprotinin, and 4 ng/ml leupeptin at pH 7.5. All procedures were done at 4 °C. The homogenate was centrifuged at $1000 \times g$ for 10 min to remove nuclei and unbroken cells. The pellet was discarded and the supernatant was centrifuged for 15 min at $10{,}000 \times g$ providing a crude mitochondrial preparation. The crude mitochondrial pellet was washed and stored at −20 °C until used. The supernatant from the $10{,}000 \times g$ spin was then centrifuged at $100{,}000 \times g$ for 1 h, this provided the crude membrane fraction (pellet) and the cytosol (supernatant). These were stored at −20 °C

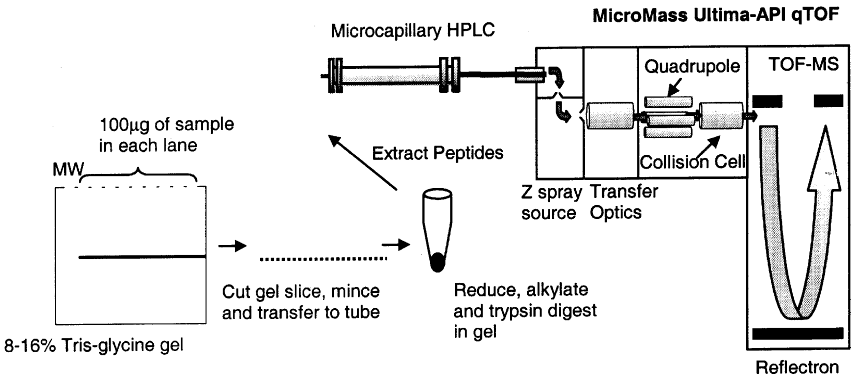


Fig. 1. Experimental outline: 100 µg of either crude neutrophil cytoplasm, mitochondrial or membrane proteins were loaded in nine wells of an 8–16% Tris–glycine gel and run for 2 h. The gel was cut into ~1 mm slices encompassing all nine wells. The slices were chopped into small pieces and proteins were reduced, alkylated and trypsin digested in gel. Peptides were extracted and each fraction injected onto the microcapillary reverse-phase HPLC column. Peptides were eluted off the column directly to the mass spectrometer. Peptides were ionized and sent through the z-spray source and transfer optics. Peptides were surveyed, meaning the quadrupole and collision cell allow all ions to pass and all peptides are detected at the TOF Software selects the five most abundant peptides for fragmentation. Fragmentation is achieved by the quadrupole allowing only the ion of choice to pass, the collision cell fragmenting the ion and the TOF detecting the fragments.

until used. Total protein concentrations were determined using the Protein Assay Kit (BioRad, Hercules CA), using a BSA standard.

2.2. Gel electrophoresis, protein alkylation and ingel trypsin digestion

Fig. 1 shows the experimental outline for protein separation, modification, digestion and analysis. One milligram of extracted protein was resuspended in a modified Laemmli buffer containing 150 mg/ml urea and 65 mM DTT and heated to 90 °C for 10 min. The sample was loaded and electrophoresed for 1.5 h at 125 V on an 8–16% Tris–glycine gel (Novex, San Diego, CA). At the end of electrophoresis the gel was stained with Gel Code Blue (Pierce, Rockford, IL) for 1 h and destained overnight in water. The gel was then cut into 37–54 slices, representing molecular weights from ~6 to 250 Kd. Each slice was cut into 1 mm cubes and placed into a siliconized microcentrifuge tube. The diced gel slices were then destained by three washes in 50:50 acetonitrile (ACN):25 mM NH_4HCO_3, followed by one wash in 80:20 ACN:25 mM NH_4HCO_3. The gel slices were then dried for 15 min in a vacuum centrifuge. To reduce disulfide bonds, the samples were rehydrated in freshly prepared 10 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCL) (Pierce, Rockford, IL) in 25 mM NH_4HCO_3, sufficient to cover the gel slices and incubated for 1 h at 56 °C. The samples were cooled to room temperature, and the TCEP-HCI solution was removed and discarded. Freshly prepared 55 mM iodoacetimide in 25 mM NH_4HCO_3 was added to the gel slices to covalently bind cysteine residues. Gel slices were incubated for 1 h at room temperature in the dark. This solution was discarded, and the slices were washed 2× with 50:50 ACN:25 mM NH_4HCO_3, followed by one wash with 80:20 ACN:25 mM NH_4HCO_3. The gel slices were dried for 15 min in a vacuum centrifuge and then rehydrated with 25 mM NH_4HCO_3. The proteins were thermally denatured at 90 °C for 20 min as described (Park and Russell, 2000, 2001). The slices were then cooled on ice for 10 min, the supernatant discarded and the gel slices were washed 2× with 50:50 ACN:25 mM NH_4HCO_3, followed by one wash with 80:20 ACN:25 mM NH_4HCO_3. The gel slices were again dried in a vacuum centrifuge. Enough proteomic grade trypsin (20 μg/ml in 25 mM NH_4HCO_3) was used to cover the gel fragments. After the gel slices were rehydrated, ACN was added so that the solution was 30% ACN (Russell et al., 2001). This ACN/trypsin solution was incubated at 37 °C overnight. The next day the samples were cooled to room temperature, and the digest solution was transferred to clean siliconized tubes. Peptides were extracted from the gel slices 2× with 50:50 ACN:5% formic acid, 1× with 15:50:35 isopropanol:ACN:5% formic acid, and 2× with 80% ACN. All extracts were combined and dried in vacuum centrifuge. The samples were stored dry at −20 °C until used.

2.3. High performance chromatography and tandem mass spectroscopy of the samples

The in-gel digest of each slice was analyzed by capillary high pressure liquid chromatography (CapLC, Waters, Milford, MA) in line with a Q-TOF Ultima API mass spectrometer (Waters, Milford, MA). An Altantis C18 NanoEase column (75μm × 100 mm) was used for peptide separation. The system was configured to concentrate and wash the injected sample on a Symmetry 300 C_{18} precolumn. Seven minutes after the start of sample loading the precolumn was switched in line with the analytical column to allow the trapped peptides to be eluted onto the analytical column. Mobil phase A was 0.1% formic acid in 5% ACN. Mobil phase B was 0.1% formic acid in 95% ACN. The gradient was 95% A for 5 min and then ramped linearly to 60% A over 85 min. Over the next 2.5 min it was ramped to 10% A and held an additional 10 min before re-equilibration of the column. The flow rate was approximately 300 nl/min. The analytical column was connected to Waters lockspray–nanospray interface on the front of the mass spectrometer. The lockspray used the peptides [Glu^1]-Fibrinopeptide B and Leucine Enkephalin as standards (Sigma, St. Louis, MO). The capillary voltage was 3500 V and was tuned for signal intensity. The five most intense ions with charge states between 2 and 4 were selected in each survey scan if they met the switching criteria. Three collision energies were used to fragment each peptide ion based on its mass to charge (*m*/*z*) values.

2.4. Protein identification

Post-run processing of the MS/MS data, which included deisotoping, mass correction via lockspray and database searching was done using ProtefinLynx Global Server 2.0 (Waters, Milford, MA). Proteins were identified using the universal protein non-redundant reference database UniRef 100 (Swiss Institute of Bioinfomatics, Basel, Switzerland) and the non-redundant protein database (National Center for Biotechnology Information, Bethesda, MD). Modifications allowed were one missed cleavage per peptide, methionine oxidation and carboxyaminomethylation. Mass accuracy of parent ions was less than 20 ppm for all spectra. MS/MS spectra with a peptide ladder score of greater than 30 were considered good sequence results.

3. Results and discussion

Neutrophil proteins from the cytoplasm, crude membrane and crude mitochondrial fractions were run on one-dimensional SDS PAGE gels. Each gel was sliced into 37–54 fragments. The proteins were reduced, alkylated, trypsin digested and extracted from the gel (Fig. 1). Each fraction was then injected onto the capillary liquid chromatography for resolution of peptides for direct analysis by mass spectrometry. The summation of all data sets resulted in greater than 1400 unique peptide spectra with a ladder score of greater than 30. The ladder score is defined as the percent of expected b and y ions from a given peptide. Approximately 1100 peptide spectra correspond to unique peptide sequences. The remaining peptide spectra either represent peptide spectra in multiple *m/z* charge states, identical peptides that are common to a family of proteins, or modifications of the peptide that occurred either in the cell or in the processing of the peptides. Multiple peptide spectra identifying the same peptide increase the confidence in the identification of the peptide. The peptide spectra were used to match proteins from the protein database (UniREF100). This allowed us to identify greater than 250 proteins (Tables 1–8). Approximately 200 proteins were identified with 2 or more quality peptide spectra (>30 ladder score), and over 40 proteins were matched with 10 or more quality peptide spectra. Although one quality MS/MS spectra has been used to identify a protein, additional peptide sequences give greater confidence in protein identification. Furthermore, additional peptide matches may be necessary to distinguish members of a protein family that may not be distinguished if the peptide sequenced is in a conserved region (e.g. BMAP proteins). The majority of these 250 proteins represent the abundant proteins in circulating bovine neutrophils.

Analysis of a whole proteome is complicated by several factors. First, the number of peptides eluting at any point in time in the online HPLC gradient is very large. Tandem mass spectrometry instruments typically will focus on the most abundant ions and needs several seconds to obtain enough data to generate useful peptide spectra; in this time many peptides go undetected. The second complication of whole proteome analysis is that different cellular components have different solubility issues. For example, two-dimensional SDS-PAGE gel analysis of membrane proteins is difficult because of their low abundance, large size and hydrophobicity (Buttner et al., 2001; Gu et al., 2003). To address these issues we chose to run cytosol, crude mitochondrial and membrane cellular fractions on one-dimensional SDS-PAGE gels. Cutting the gel into multiple slices, digesting the proteins and running each separately on the LCMS reduced the complexity of each sample enough to allow detection of lower abundance proteins. Furthermore, one-dimensional SDS-PAGE gel electrophoresis is best suited for membrane protein survey (Gu et al., 2003; Peirce et al., 2004). Comparison of our proteomic survey of bovine neutrophil to the proteomic surveys of other species is complicated by the fact that other studies have used two-dimensional SDS-PAGE gel analysis prior to mass spectrometry (Fessler et al., 2002; Piubelli et al., 2002). Our ability to identify a greater number of proteins than earlier works can be attributed to the changes in methodologies in recent years, rather than differences in species.

Most problematic to proteomic research in dairy cows is the incomplete nature of the genomic data. Proteomic analysis requires matching peptide spectra to potential peptides from known proteins. Just over 40% of the proteins identified in this study matched bovine proteins in the database. The remaining ~60%

Table 1
Metabolic proteins

Name	Function	Accession
1190005M04Rik protein	Peptide proteinase inhibitor	Q9D154
6-Phosphogluconate dehydrogenase decarboxylating	Pentose phosphate pathway	Q9DCD0
Acetyl CoA synthetase 2	Lipid transport/metabolism	NP_777171
Adenylate kinase isoenzyme 2 mitochondrial	Phosphotransferase	P08166
Aldehyde dehydrogenase mitochondrial	Ethanol utilization	P20000
Aldolase 1 A isoform	Glycolysis	Q9CPQ9
Alpha enolase	Glycolysis	Q9XSJ4
Argininosuccinate synthase	Arginine biosynthesis	P14568
ATP citrate synthase	Lipid synthesis	Q91V92
ATP synthase alpha chain mitochondrial	Produces ATP from ADP	P19483
ATP synthase beta chain mitochondrial	Produces ATP from ADP	P00829
Cc1 6	Phosphorylase activity	Q7TP15
CDP diacylglycerol inositol 3 phosphatidyltransferase	Biosynthesis of phosphatidylinositol	P70500
Cystatin C precursor	Protease inhibitor	P01035
Cytochrome b 245 heavy chain GP91-PHOX	Oxidative burst	O46522
Cytochrome b 245 light chain P22-phox	Oxidative burst	O46521
Cytochrome c1 heme protein mitochondrial	Component of cytochrome *b–d*	P00125
Cytosolic aminopeptidase P	Proteolysis and peptidolysis	Q91Y37
Dihydropyrimidine dehydrogenase NADP	Pyrimidine base degradation	Q28007
Flavoprotein subunit of succinate ubiquinone reductase	Electron transport	Q920L2
Fructose 1 6 bisphosphatase	Gluconeogenesis	P09199
Fructose bisphosphate aldolase A	Glycolysis	P04075
Fumarate hydratase mitochondrial	Tricarboxylic acid cycle	P97807
Glucose 6 phosphate 1 dehydrogenase X	Pentose phosphate pathway	Q00612
Glucose 6 phosphate isomerase	Glycolysis	P08059
Glucose phosphate isomerase 1 complex	Glycolysis	Q8C675
Glutathione peroxidase 1	Oxidative protection	P00435
Glutathione S transferase P	Conjugation of reduced glutathione	P28801
Glyceraldehyde 3 phosphate dehydrogenase	Glycolysis	P10096
Glycerol phosphate dehydrogenase 1	Glycolysis	Q8CBX6
Glycogen phosphorylase	Glycolysis	P06737
GTP AMP phosphotransferase mitochondrial	Adenylate kinase family	P08760
Hemoglobin alpha chain	Oxygen transport	P01966
Hemoglobin beta chain	Oxygen transport	P02070
Hexokinase type III	Hexokinase	P27926
Hypothetical protein FLJ10983	Carbohydrate metabolism	Q9NV22
Inositol 1 or 4 monophosphatase	Synthesis phosphatidylinositol	P20456
Lactate dehydrogenase B	Anaerobic glycolysis	Q9MYV5
Lactoperoxidase	Peroxidase	NP_536345
Leukotriene A 4 hydrolase	Hydrolyzes of leukotriene A4	P30349
Malate dehydrogenase mitochondrial	Anaerobic glycolysis	P00346
Megsin	Peptide proteinase inhibitor	Q920J5
Micromolar calcium-dependent neutral protease large subunit (Calpain 1)	Calpain activity	Q9N185
MMP9	Proteolysis and peptidolysis	Q9N282
Multifunctional protein ADE2	Purine biosynthesis	P22234
NADPH oxidase cytosolic protein p67phox	Oxidative burst	Q95L70
Neutrophil cytosol factor 1	Oxidative burst	O77774
Peptide methionine sulfoxide reductase	Oxidative protection	P54149
Peroxiredoxin 1	Antioxidant	Q9BGI4
Peroxiredoxin 5 mitochondrial precursor	Oxidative burst	Q9BGI1
Peroxiredoxin 6	Oxidative protection	O77834
Phenol sulfating phenol sulfotransferase	Sulfotransferase activity	P50227

Table 1 (*Continued*)

Name	Function	Accession
Phosphoglucomutase	Glycolysis	Q9D0F9
Phosphoglycerate mutase 1	Glycolysis	Q9DBJ1
Phosphoglycerete kinase 1	Glycolysis	Q8NI87
Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	Carbohydrate metabolism	NP_071409
Platelet activating factor acetylhydrolase IB gamma subunit	Lipase	Q15102
Polyubiquitin	Proteolysis and peptidolysis	Q28170
Proteasome	Proteolysis and peptidolysis	Q8BTU5
Proteasome activator 28 beta subunit	Proteolysis and peptidolysis	Q863Z0
Protein arginine deiminase type IV	Protein modification	088807
Protein L isoaspartate D aspartate O methyltransferase	Repair/degradation proteins	P15246
Purine nucleoside phosphorylase	Purine phosphorylase	P55859
Puromycin sensitive aminopeptidase	Proteolysis and peptidolysis	Q91VJ8
Putative phosphoglycerate mutase 3	Glycolysis	Q8N0Y7
Pyruvate kinase M1 isozyme	Glycolysis	P14618
S adenosylhomocysteine hydrolase	One-carbon metabolism	Q8HXL1
Serine threonine protein phosphatase PP1 alpha 1	Protein phosphorylation	1P08129
Seryl tRNA synthetase	Protein synthesis	Q9GMB8
Similar to glyceraldehyde 3 phosphate dehydrogenase	Glycolysis	Q862K1
Succinyl CoA ligase GDP forming alpha chain mitochondrial	Tricarboxylic acid cycle	P13086
Sulfhydryl oxidase	Electron transport	Q8TDL6
Sulfide quinone oxidoreductase mitochondrial	Mitochondrial sulfide oxidation	Q9Y6N5
Sulfide quinone reductase	Disulfide oxidoreductase	Q8BW20
Superoxide dismutase Mn mitochondrial	Oxidative protection	P41976
Synaptic vesicle membrane protein VAT 1 homolog	Alcohol dehydrogenase	Q99536
Threonyl tRNA synthetase cytoplasmic	Protein synthesis	P26639
Transaldolase	Pentose phosphate pathway	Q9EQS0
Transketolase	Pentose phosphate pathway	P29401
Triosephosphate isomerase	Glycolysis	P48500
Tryptophanyl tRNA synthetase	Protein synthesis	P17248
ubiquinol cytochrome c reductase EC 1 10 2 2 core protein I	Mitochondrial respiratory chain	P31800
Ubiquinol cytochrome c reductase complex core protein 2	Mitochondrial respiratory chain	P23004
Ubiquitin activating enzyme E1	Proteolysis and peptidolysis	O77781
UTP glucose 1 phosphate uridylyltransferase 2	Nucleotidyltransferase activity	Q07130
Vacuolar ATP synthase catalytic subunit A ubiquitous isoform	Vacuolar ATPase	Q29048

Bold type indicates the peptides that matched a bovine sequence in the database.

identified were proteins homologous to either human, mouse or rat. Because the analysis software only allows for limited divergence of the peptide sequence from that reported in the protein database, peptide spectra that represent peptides in polymorphic regions of unsequenced genome will go unidentified. The full power of proteomic research in dairy cows will be realized only upon completion of the bovine genome.

Proteins were grouped into basic functional groups (Fig. 2). Nearly 35% of the peptides identified were enzymes involved in basic cellular metabolic pathways (Table 1). Since circulating neutrophils have such a short half-life and a singular purpose, it should not be surprising that so much of the proteome should be dedicated to a few general functional groups. Because circulating neutrophils are posed to respond to inflammation very quickly, it would seem reasonable that metabolic enzymes required for the primary functions of the neutrophil would be abundant. Consistent with that hypothesis the majority of the enzymes involved in the pentose phosphate pathway and gluconeogenesis that would be required for NADPH generation, important for neutrophil oxidative burst, were identified. In addition, most of the enzymes involved in glycolysis and the generation of ATP, which would provide the energy necessary for cell mobility and transmigration into inflamed tissue, were found.

Proteins involved in cell structure/mobility (Table 2) or immune functions (Table 3) make up

Table 2
Cell mobility

Name	Function	Accession
Actin beta	Actin	Q7M3B0
Actin, cytoplasmic 1	Actin	P60712
Actnl protein	Cross-linking	AAH03232
Adenylyl cyclase associated protein 1	Actin capping	NP_006358
ARP2 3 complex 16 kDa subunit	Control of actin polymerization	O15511
ARP2 3 complex 21 kDa subunit	Control of actin polymerization	O15145
ARP2 3 complex 34 kDa subunit	Control of actin polymerization	O15144
ARP2 3 complex 41 kDa subunit	Control of actin polymerization	O15143
ARPC4 protein	Control of actin polymerization	NP_005709
Beta actin	Motor activity	Q9TTW4
CD44 antigen precursor	Cell adhesion	Q29423
Coactosin like protein	Actin capping	Q9CQI6
Cofilin non-muscle isoform	Control of actin polymerization	P18760
Coronin 1C	Highly motile cells	Q9ULV4
Coronin like protein p57	Highly motile cells	Q92176
Ezrin	Connect membrane to cytoskeleton	P31976
F actin capping protein beta subunit	Actin capping	P79136
Fibrinogen beta chain precursor	Precursor to fibrin	P02676
Fibrinogen gamma B chain precursor	Precursor to fibrin	P12799
Filamin 1	Actin binding	Q95LH5
Filamin A	Actin binding	Q8WMQ6
Gelsolin precursor plasma	Actin binding	P20305
Hypothetical protein	Motor activity	Q8N532
Hypothetical protein DKFZp451J0218	Motor activity	Q86T83
Intercellular adhesion molecule 3 precursor	Cell adhesion	Q28125
L plastin	Actin binding	P13796
MKIAA1027 protein	Actin binding	Q80TM2
Moesin	Connect membrane to cytoskeleton	O35763
MYH14 protein	Myosin	Q8WV23
Myh9 protein	Myosin	AAH06075
Peripherin	Filament protein	P41219
Profilin 1	Actin binding	P02584
Radixin	Actin binding	NP_002897
Ras GTPase activating like protein IQGAP1	Assembly scaffold	P46940
Similar to tropomyosin 3 gamma	Interactions with actin	Q8K0Z5
Smooth muscle myosin heavy chain isoform SM1A	Motor activity	Q63861
T plastin	Actin binding	Q63598
Thrombospondin 1	Cell adhesion	Q28194
Tropomyosin	Motor activity	Q63599
Tropomysin cytoskeletal type	Interactions with actin	Q29219
TUBB1 human beta tubulin 1 class VI	Microtubule	Q9H4B7
Tubulin beta 1 chain	Microtubule	P07437
tubulin, beta, 4	Microtubule	AAP36356
Ubiquitous tropomodulin	Control of actin polymerization	Q9NYL9
Vasodilator stimulated phosphoprotein	Actin binding	P50552
Vimentin	Filament protein	P48616
Vinculin	Connect membrane to cytoskeleton	P12003

Bold type indicates the peptides that matched a bovine sequence in the database.

the next two most abundant groups of peptides with approximately 15% of the total peptide spectra each. In addition to the many proteins that make up the cytoskeleton of the cell, we have found proteins predicted to link the immunological function of a neutrophil with cytoskeletal rearrangement. Coronin 1C and Coronin-like protein (Table 2) are actin-associated proteins that have been shown to be

Table 3
Immune proteins

Name	Function	Accession
Antibacterial peptide BMAP 27 precursor	Antimicrobial	P54228
Antibacterial peptide BMAP 28 precursor	Antimicrobial	P54229
Antibacterial peptide BMAP 34 precursor	Antimicrobial	P56425
Bactenecin 5 precursor	Antimicrobial	P19660
Bactenecin 7 precursor	Antimicrobial	P19661
Calgranulin B	Antimicrobial	P28783
Calgranulin C	Antimicrobial	P79105
92 kDa type IV collagenase precursor	Cleaves collagen	P52176
Complement C4 precursor	Complement	P01030
Cyclic dodecapeptide precursor	Antimicrobial	P22226
Elastase	Proteolysis and peptidolysis	Q9GME1
Eosinophil peroxidase precursor	Peroxidase	P11678
High affinity immunoglobulin epsilon receptor gamma subunit precursor	Fc receptor	Q9BDR7
Indolicidin precursor	Antimicrobial	P33046
Lactotransferrin precursor	Antimicrobial	P24627
Leukocyte elastase inhibitor	Regulates elastase	P80229
Lysosomal alpha mannosidase precursor	Granule	Q29451
Macrophage migration inhibitory factor	Macrophage migration	P80177
Myeloperoxidase precursor	Oxidative burst	P11247
Peptidoglycan recognition protein precursor	Antimicrobial	Q8SPP7

Bold type indicates the peptides that matched a bovine sequence in the database.

important for phagocytosis and formation of the phagocytic vacuole. In addition, coronin is attached to p40-phox, a member of the NADPH oxidase complex, thus linking the neutrophil functions of oxidative burst with phagocytosis (Grogan et al., 1997). Similarly, Moesin (Table 2) has been shown to bind actin and other proteins such as CD44 (Table 2), p40-phox and p47-phox (Wientjes et al., 2001).

The granules of neutrophils contain proteins that have important antimicrobial functions (Zanetti, 2004). For example, bovine neutrophils have seven members of the cathelicidin family: cyclic dodecapeptide, bactenecin 5, bactenecin 7, indolicidin, bovine myeloid antibacterial peptide (BMAP)-27, BMAP-28 and BMAP-34 (Hoverprot Database, Lyon, France). Upon degranulation of neutrophils the cathelicidins are cleaved by a protease such as elastase to generate protein fragments that have lytic activity against fungi and both gram-positive and gram-negative bacterium (Zanetti, 2004). Our analysis of abundance neutrophil proteins found all seven of the cathelicidin members (Table 3). In addition, we found other proteins found within neutrophil granules, such as lysosomal alpha mannosidase, elastase, lactotransferrin and collagenase (Smith, 2000).

One of the major antimicrobial mechanisms of the neutrophil is its ability to generate free radicals both within a phagosome and extracellularly (Vignais, 2002). The protein complex, NADPH oxidase, transforms oxygen into a superoxide anion. The NADPH oxidase complex proteins are present in two cellular compartments: those that are membrane bound and those found in the cytosol. The catalytic component of NADPH oxidase, also known as flavocytochrome b_{558}, consists of two membrane bound proteins referred to as gp91phox and p22phox. Association of cytosolic proteins with the flavocytochrome b_{558} causes the functional activation of the complex. In addition to the two flavocytochrome b_{558} proteins, many of the cytosolic proteins that comprise the NADPH oxidase complex (p67phox, Rap1A, Rad, Rac 2 and S100A9; Doussiere et al., 2002; Vignais, 2002) were identified in this study. Interestingly, we could not find the p47phox protein that has been shown to be necessary to activate flavocytochrome b_{558} in a cell free assay (Abo et al., 1992). However, a recent report suggests a HMG box containing protein HBP1 acts as a transcriptional repressor for p47phox (Berasi et al., 2004). We found in our circulating neutrophil sample an HMG box containing protein known as high mobility group protein 1 (Table 4). It is unknown

Table 4
DNA/protein synthesis

Name	Function	Accession
10 days embryo cDNA RIKEN full length enriched library clone 2610017D13 full insert sequence	Chromosome	Q9D0H3
145 kDa translational inhibitor protein	Protein synthesis	P52758
60S ribosomal protein L10a	Ribosomal	P53027
60S ribosomal protein L12	Protein synthesis	P30050
60S ribosomal protein L7	Ribosomal	P18124
Activated RNA polymerase II transcriptional coactivator p15	Transcription	P53999
BA317E16 2	Chromosome	Q96QV6
C367G8 3	Protein synthesis	Q9BR02
Elongation factor 1 alpha 1	Protein synthesis	1P04720
Eukaryotic translation initiation factor Eif4a2	Protein synthesis	Q9XT93
Glycogen debranching enzyme	Glycogen degradation	P35573
Heterogeneous nuclear ribonucleoprotein A2 B1 B0	Nucleic acid binding	Q8CJ71
High mobility group protein 1	Transcription	P10103
Histone 3, H2ba	Chromosome	NP_084358.1
Histone 4	Chromosome	2P02304
Histone H1 1	Chromosome	P02253
Histone H1 5	Chromosome	P43276
Histone H2A z	Chromosome	3P17317
Histone H2B a g k	Chromosome	1P02278
HP1 BP74 protein	Transcription	Q9UHY0
Hypothetical protein	Protein synthesis	Q8VC94
Hypothetical protein FLJ10903	Chromosome	Q9NV63
MACROH2A2	Chromosome	Q8CCK0
Microsomal signal peptidase 25 kDa subunit	Protein synthesis	Q9CYN2
mRNA cleavage factor I 25 kDa subunit	Transcription	O43809
Polypyrimidine tract binding protein	Transcription	Q8WN55
Prohibitin	Regulating proliferation	P35232
Ribosomal protein L8	Ribosomal	Q9BWQ9
Small nuclear ribonucleoprotein Sm D1	Transcription	1P13641
Splicing factor	Nucleic acid binding	Q8C3H6

Bold type indicates the peptides that matched a bovine sequence in the database.

whether this protein affects p47phox transcription. In addition, we identified superoxide dismutase and myeloperoxidase, which further transform superoxide anions into hydrogen peroxide and hypochlorite, respectively (Smith, 2000).

Heat shock proteins (HSPs) are a group of ubiquitous conserved proteins whose expression is modulated by environmental or physiological stresses. These proteins play an essential role in assisting nascent protein folding and clearing of misfolded proteins. In addition, HSPs have been shown to play an important regulatory role of the programmed cell death mechanisms. For example HSP-70 overexpression has been shown to prevent steps in the apoptitic pathway preventing important protease activating and nuclear and morphological changes associated with apoptosis (Parcellier et al., 2003). HSPs identified in this study that have a described role in apoptosis are HSP-70 protein 1, HSP-70 protein 2 and HSP-90 beta (Table 5). Interestingly, a study involving major burn patients showed an increased expression of HSP-27, HSP-60, HSP-70 and HSP-90 in neutrophils subsequent to injury that correlated with increased oxidative activity and reduced apoptosis (Ogura et al., 2002). Because of HSPs involvement in the regulation of the apoptitic pathway, these proteins are of great interest in the study of neutrophil homeostasis.

Critical to neutrophil function are the mechanisms responsible for signal transduction, which enable the cell to respond to environmental stimuli. The 14-3-3 protein family is a group of proteins that have been suggested to play an important regulatory role in signal transduction pathways that regulate exocytosis,

Table 5
Binding and chaperone proteins

Name	Function	Accession
BiP protein	Chaperone	Q9UK02
Calnexin precursor	Protein folding	P35564
Endoplasmic reticulum protein ERp29 precursor	Protein folding	NP_006808
Ferritin light chain	Binds iron	O46415
FK506 binding protein 1A	Protein folding	P18203
Haptoglobin	Binds hemoglobin	Q9MYV8, Q7M365, Q8SPS7
Heat shock 70 kDa protein 1	Protein folding	Q27975
Heat shock 70 kDa protein 2	Protein folding	Q27965
Heat shock cognate 71 kDa protein	Chaperone	P19120
Heat shock protein 1A	Protein folding	P55063
Heat shock protein 90 beta	Protein folding	Q865A1
Heat shock protein beta	Chaperone	O14942
Peptidyl prolyl cis–trans isomerase A	Protein folding	P04374
Peptidyl prolyl cis–trans isomerase B precursor	Protein folding	P80311
Phosphatidylethanolamine binding protein	Binds small molecules	P13696
Protein disulfide isomerase A3 precursor	Protein folding	P38657
Serotransferrin precursor	Binds iron	Q29443
Stress 70 protein mitochondrial precursor	Chaperone	P38646
Swiprosin 1	Binds calcium	Q96C19
T complex protein 1 theta subunit	Protein folding	P50990

Bold type indicates the peptides that matched a bovine sequence in the database.

Table 6
Cell signaling

Name	Function	Accession
14-3-3 protein beta alpha	Activates protein kinase C	P29358
14-3-3 protein gamma	Activates protein kinase C	P29359
14-3-3 protein tau	Activates protein kinase C	P35216
14-3-3 protein zeta delta	Activates protein kinase C	1P29312
cAMP dependent protein kinase type I alpha regulatory chain	Kinase	P00514
Cell division control protein 42 homolog	Cell cycling	2P21181
Guanine nucleotide binding protein G i alpha 1 subunit	G protein	1P04898
Inhibitory GTP binding protein subunit alpha 2	G protein	Q95KM8
Integrin beta 2 precursor CD18	Integrin	P32592
Leukocyte common antigen precursor CD45	Ptpase	P08575
Leukocyte integrin alpha M chain CD11B	Integrin	Q8HY41
Protein tyrosine phosphatase non-receptor type 6	Ptpase	P29351
Rab GDP dissociation inhibitor alpha	Regulates Rab	P21856
Ras related C3 botulinum toxin substrate 1	Regulates NADPH	2P15154
Ras related C3 botulinum toxin substrate 2	Regulates NADPH	Q9TU25
Rho associated coiled coil forming protein kinase p160 ROCK 1	Intracellular signaling	P70335
Rho GDP dissociation inhibitor 1	Regulates Rho	P19803
Rho GDP dissociation inhibitor 2	Regulates Rho	Q9TU03
Rho related GTP binding protein RhoG	G Protein	P35238
Septin 6	Cell cycling	Q14141
Signal transducer and activator of transcription 1	Transcription activator	Q9D323
Similar to phospholipase C gamma 2	Intracellular signaling	Q8VE69
WD repeat domain 1	Intracellular signaling	Q9DC08

Bold type indicates the peptides that matched a bovine sequence in the database.

Table 7
Trafficking/transport

Name	Function	Accession
ABC transporter ABCA6	ABC transporter	Q8N139
Annexin A1	Exocytosis	P46193
Annexin A11	Exocytosis	P27214
Annexin A3	Exocytosis	O35639
Annexin A4	Exocytosis	P13214
Annexin A5	Exocytosis	P81287
Chloride intracellular channel protein 1	Ion	O00299
Copine I	Membrane	Q99829
Copine II	Membrane	Q96FN4
Copine III	Membrane	O75131
Copine IV	Membrane	Q96A23
Epsilon 1 globin	Oxygen	O88752
Erythrocyte band 7 integral membrane protein	Cation conductance	P27105
Flotillin 1	Vasicular	NP_032053
Glucose transporter 14 short form	Sugar	Q8TDB9
GTP binding nuclear protein RAN	Nucleocytoplasmic transport	1P17080
Importin beta 1 subunit	Protein	Q14974
Ras related protein Rap 1A	Regulates Ras	3P10113
Ras related protein Rab 1B	Protein	Q9H0U4
Ras related protein Rab 2A	Protein	P08886
Ras related protein Rab 5C	Vasicular	P51148
Ras related protein Rab 7	Vasicular	P51149
Serum albumin precursor	Ion	P02769
Solute carrier family 2 facilitated glucose transporter member 3	Glucose	P58352
Valosin-containing protein	Vasicular	NP_009057
Voltage dependent anion channel 2	Anion transport	Q9MYV7

Bold type indicates the peptides that matched a bovine sequence in the database.

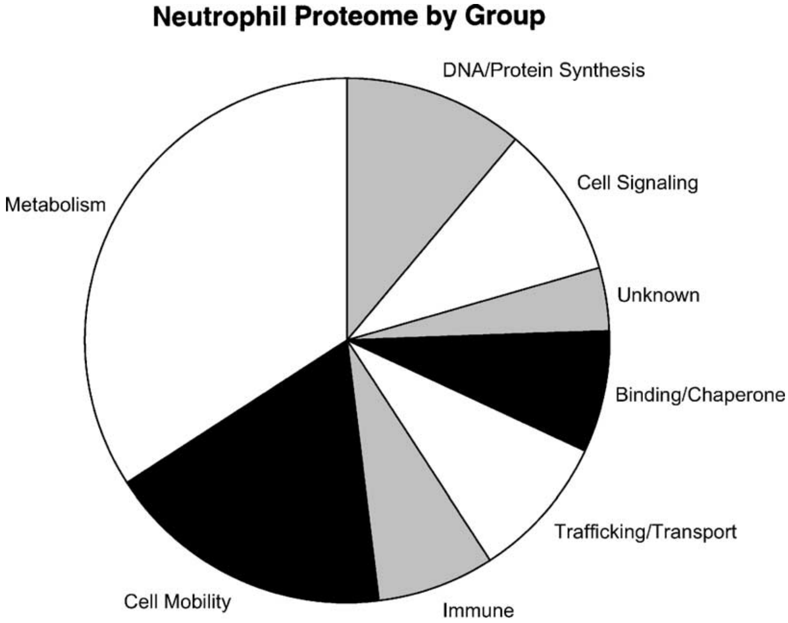
cell cycle regulation and apoptosis. We have found four members of the 14-3-3 protein family expressed by the bovine neutrophil (Table 6). The primary function of the 14-3-3 zeta protein has been determined to be the inhibition of apoptosis (Xing et al., 2000). The observation of HSPs and the 14-3-3 proteins highlight the importance of apoptosis in the regulation of life and function of the neutrophil.

Table 8
Unknown

Name	Accession
Apoa I binding protein precursor	Q8NCW5
Bm009	Q9NZE7
Butyrophilin	O46535
Cyfip2	Q924D3
Hypothetical protein	Q9UG05
Hypothetical protein FLJ11197	Q9NUQ9
IFN response element binding factor 2	Q06477
Osteoclast stimulating factor	Q8MJ50
SH3 domain binding glutamic acid rich like protein 3	Q91VW3
Similar to stomatin peptide	Q8TAV4
TNF induced protein GG2 1 homolog	Q8BTH4

Bold type indicates the peptides that matched a bovine sequence in the database.

A class of proteins critical to intracellular trafficking and transport are known as the Rab proteins. Rab proteins are a large group of small GTPases that are part of the Ras protein superfamily. The function of Rab proteins is to regulate the transport between organelles. Specific Rab proteins are localized to distinct intracellular compartments where they function as location markers throughout the cell. The activity of the Rab proteins is controlled by its GTPase function, being bound to GTP in its active state and GDP in the inactive. The Rab proteins have been shown to function in the tethering/docking of vesicles to their target vesicle and also have been implicated in vesicle budding and regulation of vesicle movement along the cytoskeletal filaments (Zerial and McBride, 2001). Two ER–golgi intermediate proteins, Rab1B and Rab2A were identified as well as the early endosomal protein, Rab5C and the late endosomal



Group	Number of MSMS Spectra	Number of Proteins	Percent of Total Proteins
Metabolism	486	91	34.1%
Cell Mobility	273	48	18.0%
Immune	211	19	7.1%
DNA/Protein Synthesis	108	30	11.2%
Binding/Chaporone	86	20	7.5%
Cell Signaling	126	25	9.4%
Trafficking/Transport	90	24	9.0%
Unknown	24	10	3.7%

Fig. 2. Neutrophil proteome by functional groups: neutrophil proteins identified were divided into eight functional groups based on known functions or sequence based on domain similarity. The table lists the functional group, number of MS/MS spectra found in each group, number of proteins in each group and percent of the total protein that is from each group.

protein Rab7 (Table 7). It is probable that Rab proteins play an important role in neutrophil function such as phagocytosis and granule organization and release.

Table 8 contains a list of proteins with unknown functions. The 250 proteins found in this study represent all the basic functions of a neutrophil. This study provides the baseline information at the molecular level regarding the proteome of the bovine neutrophil.

4. Conclusions

Mass spectrometry allows for the survey of the proteins expressed in cells or tissues. Due to the importance neutrophils play as the first line of defense to invading pathogens and the correlation between depressed neutrophil function and mastitis in dairy cows we chose circulating neutrophils as a cell type for the first phase of analysis of its proteome. We have identified over 250 proteins in the bovine neutrophil proteome.

The identification of 250 proteins in the bovine neutrophil proteome represents only the first phase in studies planned to apply proteomics to issues important to dairy cow health. The next stage of this research will focus on the identification of proteins differentially regulated during the immunosuppression of neutrophil function in periparturient dairy cows.

References

Abo, A., Boyhan, A., West, I., Thrasher, A.J., Segal, A.W., 1992. Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21racl, and cytochrome *b*-245. J. Biol. Chem. 267, 16767–16770.

Aebersold, R., Mann, M., 2003. Mass spectrometry-based proteomics. Nature 422, 198–207.

Berasi, S.P., Xiu, M., Yee, A.S., Paulson, K.E., 2004. HBP1 repression of the p47phox gene: cell cycle regulation via the NADPH oxidase. Mol. Cell Biol. 24, 3011–3024.

Boussac, M., Garin, J., 2000. Calcium-dependent secretion in human neutrophils: a proteomic approach. Electrophoresis 21, 665–672.

Buttner, K., Bemhardt, J., Scharf, C., Schmid, R., Mader, U., Eymann, C., Antelmann, H., Volker, A., Volker, U., Hecker, M., 2001. A comprehensive two-dimensional map of cytosolic proteins of *Bacillus subtilis*. Electrophoresis 22, 2908–2935.

Doussiere, J., Bouzidi, F., Vignais, P.V., 2002. The S100A8/A9 protein as a partner for the cytosolic factors of NADPH oxidase activation in neutrophils. Eur. J. Biochem. 269, 3246–3255.

Fessler, M.B., Malcolm, K.C., Duncan, M.W., Worthen, G.S., 2002. A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase. J. Biol. Chem. 277, 31291–31302.

Grogan, A., Reeves, E., Keep, N., Wientjes, F., Totty, N.F., Burlingame, A.L., Hsuan, J.J., Segal, A.W., 1997. Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. J. Cell Sci. 110 (Pt 24), 3071–3081.

Gu, S., Chen, J., Dobos, K.M., Bradbury, E.M., Belisle, I.T., Chen, X., 2003. Comprehensive proteomic profiling of the membrane constituents of a *Mycobacterium tuberculosis* strain. Mol. Cell Proteomics 2, 1284–1296.

Kehrli Jr., M.E., Nonnecke, B.J., Roth, J.A., 1989. Alterations in bovine neutrophil function during the periparturient period. Am. J. Vet. Res. 50, 207–214.

Mehrzad, J., Dosogne, H., Meyer, E., Heyneman, R., Burvenich, C., 2001. Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. J. Dairy Res. 68, 399–415.

Mollinedo, F., Borregaard, N., Boxer, L.A., 1999. Novel trends in neutrophil structure, function and development. Immunol. Today 20, 535–537.

Ogura, H., Hashiguchi, N., Tanaka, H., Koh, T., Noborio, M., Nakamori, Y., Nishino, M., Kuwagata, Y., Shimazu, T., Sugimoto, H., 2002. Long-term enhanced expression of heat shock proteins and decelerated apoptosis in polymorphonuclear leukocytes from major burn patients. J. Burn Care Rehabil. 23, 103–109.

Paape, M., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C., 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J. Mammary Gland Biol. Neoplasia 7, 109–121.

Paape, M.I., Bannerman, D.D., Zhao, X., Lee, J.W., 2003. The bovine neutrophil: structure and function in blood and milk. Vet. Res. 34, 597–627.

Parcellier, A., Gurbuxani, S., Schmitt, E., Solary, E., Garrido, C., 2003. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. Biochem. Biophys. Res. Commun. 304, 505–512.

Park, Z.Y., Russell, D.H., 2000. Thermal denaturation: a useful technique in peptide mass mapping. Anal. Chem. 72, 2667–2670.

Park, Z.Y., Russell, D.H., 2001. Identification of individual proteins in complex protein mixtures by high-resolution, high-mass-accuracy MALDI TOF-mass spectrometry analysis of in-solution thermal denaturation/enzymatic digestion. Anal. Chem. 73, 2558–2564.

Peirce, M.J., Wait, R., Begum, S., Saklatvala, J., Cope, A.P., 2004. Expression profiling of lymphocyte plasma membrane proteins. Mol. Cell Proteomics 3, 56–65.

Piubelli, C., Galvani, M., Hamdan, M., Domenici, E., Righetti, P.G., 2002. Proteome analysis of rat polymorphonuclear leukocytes: a two-dimensional electrophoresis/mass spectrometry approach. Electrophoresis 23, 298–310.

Roth, J.A., Kaeberle, M.L., 1981. Evaluation of bovine polymorphonuclear leukocyte function. Vet. Immunol. Immunopathol. 2, 157–174.

Russell, W.K., Park, Z.Y., Russell, D.H., 2001. Proteolysis in mixed organic–aqueous solvent systems: applications for peptide mass mapping using mass spectrometry. Anal. Chem. 73, 2682–2685.

Shuster, D.E., Lee, E.K., Kehrli Jr., M.E., 1996. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. Am. J. Vet. Res. 57, 1569–1575.

Smith, G.S., 2000. Neutrophils. In: Feldman, B.F., Zinkl, J.G., Jain, N.C. (Eds.), Schalm's Veterinary Hematology. Lippincott Williams & Wilking, Philadelphia, pp. 281–296.

Vignais, P.V., 2002. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell Mol. Life Sci. 59, 1428–1459.

Wientjes, F.B., Reeves, E.P., Soskic, V., Furthmayr, H., Segal, A.W., 2001. The NADPH oxidase components p47(phox) and p40(phox) bind to moesin through their PX domain. Biochem. Biophys. Res. Commun. 289, 382–388.

Xing, H., Zhang, S., Weinheimer, C., Kovacs, A., Muslin, A.J., 2000. 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. EMBO J. 19, 349–358.

Yates, J.R., 2004. Mass spectral analysis in proteomics. Annu. Rev. Biophys. Biomol. Struct. 33, 297–316.

Zanetti, M., 2004. Cathelicidins, multifunctional peptides of the innate immunity. J. Leukoc. Biol. 75, 39–48.

Zerial, M., McBride, H., 2001. Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. 2, 107–117.

Zychlinsky, A., Weinrauch, Y., Weiss, J., 2003. Introduction: forum in immunology on neutrophils. Microb. Infect. 5, 1289–1291.